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ISSN 0792 - 156X

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PUBLISHER:

Israeli Journal of Aquaculture - BAMIGDEH -  
Kibbutz Ein Hamifratz, Mobile Post 25210,  
ISRAEL

Phone: + 972 52 3965809

<http://siamb.org.il>

## Hemorrhage Disease of Cultured Tra Catfish (*Pangasianodon hypophthalmus*) in Mekong Delta (Vietnam)

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Key words: bacteria, *Pangasianodon hypophthalmus*, *Aeromonas hydrophila*, *Clostridium* sp.,  
*Edwardsiella ictaluri*, reovirus, transmission electron microscopy, Mekong Delta, Vietnam

### Abstract

This study investigated hemorrhage disease in cultured tra catfish (*Pangasianodon hypophthalmus*) from An Giang, Ben Tre, Can Tho, and Vinh Long provinces in the Mekong Delta (Vietnam). The disease is characterized by internal organ necrosis, white (spot) nodules in the liver, kidney, and spleen, and petechial hemorrhages on the tail, fins, and abdomen. Some fish have exophthalmus (pop eye), a reddish and swollen anus, and yellowish fluid in the peritoneal cavity. Moribund fish lose their appetite and swim at the surface. Bacteria isolated from the diseased fish consisted of *Aeromonas hydrophila* (38.8%), *A. sobria* (4.1%), *A. caviae* (2.0%), *Edwardsiella ictaluri* (4.1%), and a gram positive, anaerobic bacteria, *Clostridium* sp. (40.8%). Histological analyses showed necrotic cells and intranuclear, randomly-arranged, straight rod cells (1.0-1.5 x 3.0-4.0 µm) concentrated in the ulcers. Challenge test with *A. hydrophila* induced external signs of hemorrhagic disease. Challenge test with *Clostridium* sp. confirmed the presence of the bacteria in infected tissues with development of white nodules similar to those in naturally-infected fish. Fish challenged with *E. ictaluri* exhibited gas bubbles in the stomach and gut with a foul smell. Reovirus-like particles were seen by transmission electron microscopy. Further study is needed to determine the role of each pathogen alone and together with others in the pathogenesis of hemorrhage disease of tra catfish.

### Introduction

Tra catfish (*Pangasianodon hypophthalmus*) is popularly produced in southern Vietnam. It has been an important export aquaculture product in Vietnam for ten years. It was exported to 75 countries with a total value of US\$700 million in 2006 and is projected to reach one billion US\$ in 2007. The biggest market is the European Union, then Eastern Europe, North America, and other countries. Intensive tra catfish culture in the Mekong Delta has been hampered by disease outbreaks causing heavy losses. The disease occurred in the rainy season and spread rapidly throughout the population with mortality ranging 20-80%.

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Disease signs consist of petechial hemorrhages on the tail, fins, and abdomen. Some fish have exophthalmus (pop eye), a reddish and swollen anus, and yellowish fluid in the peritoneal cavity. Hemorrhage alongside the ventral fin base is also observed. The internal organs show a swollen kidney, spleen, and liver with many necrotic nodules (white spots). Moribund fish lose their appetite and swim at the surface. Bacteria may be the cause of the disease.

Under natural conditions, infection with *Aeromonas hydrophila* is probably a minor problem but, in intensive fish farming, the common occurrence of this pathogen relates to stress conditions or factors (Roberts, 1993). *Aeromonas hydrophila*, as well as *Vibrio* sp. and *Pseudomonas*, has been reported to be the cause of infectious dropsy in hybrid catfish having a swollen abdomen, swollen gills, and hemorrhages (AAHRI, 1995). It was also reported as a pathogen in Chinese catfish. Affected fish usually develop a grey to black color, may have small red sores on the body and a swollen abdomen or swelling on one or both sides of the body just behind the head (CTSA, 1996). *Aeromonas hydrophila* and *A. jandaei* are European eel pathogens and infections may occur by waterborne transmission (Esteve et al., 1993).

*Edwardsiella ictaluri* is the causative agent of enteric septicemia of catfish (ESC) which results in high mortality in channel catfish (Austin and Austin, 1999) and of a bacterial disease in walking catfish, *Clarias batrachus* (L.) in Thailand (Kasornchandra et al., 1987). It was reported to cause bacillary necrosis of *Pangasius* in tra catfish, typified by multifocal irregular white nodules of varying sizes on several organs including the liver, spleen, and kidney (Crumlish, 2002). This disease is usually accompanied by hemorrhagic clinical signs in all stages of fish.

*Clostridium botulinum* type E was reported in salmon and other marine fish in the Pacific Northwest (Craig et al., 1968). *Clostridium botulinum* was reported as the pathogen of fish botulism among rainbow trout in Britain (Cann and Taylor, 1982).

Viruses have been associated with hemorrhagic disease in channel catfish, *Ictalurus punctatus*. Channel catfish virus (CCV) is the causative agent of an acute hemorrhagic infection that typically results in high mortality up to 100% of young-of-the-year. In one of the earliest research reports on channel catfish virus disease (CCVD), the virus was isolated from four of five epizootics involving very young channel catfish, but was not found in sub-adult or adult channel catfish (Fijan et al., 1970). Another virus, channel catfish reovirus, was first isolated in 1982 (Amend et al., 1982, 1984). However, mortality was not severe and the sole pathological change was hyperplasia of the gill lamellae with multifocal fusion. Both viruses were reported in the USA (Wolf, 1988).

At present, hemorrhage disease of tra catfish is a serious problem. Outbreaks are not specific and are usually associated with other clinical signs. The aim of this study was to identify the pathogen(s) associated with the hemorrhage disease of tra catfish.

### Materials and Methods

**Fish sampling.** Diseased tra catfish manifesting varying clinical signs of hemorrhage disease were collected from pond and cage culture farms in the Mekong Delta during disease outbreaks associated with high mortality in May-December, 2007. Samples (50-500 g/fish) were collected from backyard tra catfish cultures in the provinces of An Giang (17 samples), Ben Tre (7 samples), Vinh Long (11 samples), and Can Tho (14 samples).

**Histopathology.** Tissue samples from the liver, kidney, and spleen of the sampled fish were fixed in Bouin's solution for 24 h for routine tissue processing. The tissues were dehydrated in an alcohol series (70-100%), embedded in paraffin wax, and cut into sections of 5-6 µm thickness using a rotary microtome (Leica). The tissue sections were stained with either hematoxylin and eosin (Ferguson, 2006) or Wright-Giemsa for observation by light microscopy.

**Bacteria isolation and identification.** Samples from the liver, kidney, and spleen were aseptically streaked onto blood agar plates for isolation of *Aeromonas* and *Edwardsiella*, on Rimler-Shotts agar for *Aeromonas* isolation, and on Mac Conkey agar for *Edwardsiella* isolation, then

incubated at 30°C for 18-24 h. Predominant colonies were subcultured into pure culture for subsequent identification using Gram stain, morphology, motility, oxidation/fermentation, and oxidase, catalase, and biochemical tests using API 20E. Identification of bacterial isolates was based on Bergey's Manual of Determinative Bacteriology (Holt et al., 1994).

Tissue samples were heated to 75°C for 10 min to kill vegetative cells and aerobic bacteria. Anaerobic bacteria were then isolated by strict anaerobic method onto Wilson-Blair agar base incubated in anaerobic jars for 24-48 h at 30°C.

**Virus isolation.** Tissue samples of the liver, spleen, and kidney were homogenized using a sterile mortar and pestle, then diluted in Hank's balance salt solution. Bluegill fry (BF-2) cells were grown in Leibovitz medium with 10% fetal bovine serum and antibiotics (100 IU/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin), incubated at 25°C. Tissue filtrates were inoculated into 24-well plates with BF-2 cells in duplicates of ten-fold dilutions and incubated at 27°C. The wells were checked daily for 7 days to observe for cytopathic effects (CPE). Negative wells were blind passaged for 7 more days.

**Transmission electron microscopy (TEM).** Infected tissues were immersed in a fixative consisting of 3% paraformaldehyde and 4% glutaraldehyde in 0.2 M cacodylate at 4°C. The cells were post-fixed in 1% osmium tetroxide in the 0.1M cacodylate buffer for one h at 25°C. The fixed cells were dehydrated in an ethanol series and embedded in epoxy resin. Embedded samples were sectioned with an ultramicrotome, stained with uranyl acetate-lead citrate, and examined using a transmission electron microscope at magnifications of 3,000-600,000 times.

**Fish challenge tests.** Tra catfish (25-30 g) were collected from An Giang province and stocked in 150-l tanks at room temperature (about 30°C). The fish were examined and found to be pathogen-free. They were fed and observed for 7 days before the challenge experiment to ensure that the fish were healthy. Three sets of 30 test fish were challenged by intraperitoneal injection with 0.1 ml of 10<sup>6</sup> colony forming units (CFU)/ml bacterial suspensions of either *A. hydrophila* or *E. ictaluri* or *Clostridium* sp. Another set of 30 fish served as controls and were injected with saline solution (0.85% NaCl). Abnormal behavior and mortality of the test fish were recorded for 7 days. Moribund fish with clinical signs were collected for bacterial re-isolation.

## Results

**Fish samples.** A total of 49 diseased fish were collected from the four provinces in Vietnam (Table 1). Bacteria were isolated from all 49 fish; viruses were isolated from 39 pooled fish samples. Almost all the diseased fish had been treated with antibiotics (sulfamethoxazole + trimethoprim or florphenicol or cefalexin).

Table 1. Tra catfish sample collection in Vietnam, May-December 2007.

Month	No. fish	Sampling site (province)
May	7	An Giang
June	14	Can Tho
July	11	Vinh Long
August	7	Ben Tre
December	10	An Giang
Total	49	

**Clinical signs.** Disease outbreaks caused high mortality among fish cultured in cages or ponds. External clinical signs included hemorrhage in the abdomen, the caudal peduncle, and fin bases, especially in the ventral fins. The peritoneal cavity contained yellowish fluid, gas bubbles in the stomach/gut, necrotic lesions, and white nodules (spots) in the liver and kidney.

The fish were classified into four types, based on clinical signs. Type 1 included 13 fish (26.5%) that exhibited gross signs of hemorrhage on the fins, caudal peduncle, and around the eyes and mouth, as well as exophthalmus. Type 2 included 9 fish (18.4%) with yellow fluid in the abdominal cavity. In some cases, a bad smell and gas bubbles exuded from the stomach/gut and there were light hemorrhages on the mouth and around the eyes. Type 3 included 15 fish

(30.6%) that manifested hemorrhages on the mouth, around the eyes (or exophthalmus), caudal peduncle, and fins, especially alongside the ventral fin base. Internal organs showed swollen kidney, spleen, and liver with many necrotic nodules (white spots). The abdominal cavity contained yellow fluid which in some cases exuded a bad smell and gas bubbles in the stomach/gut. Type 4 included 12 fish (24.5%) with hemorrhagic signs alongside the ventral fin base and many necrotic nodules (white spots) in the kidney, spleen, and liver.

**Bacteria identification.** *Aeromonas hydrophila* and *Clostridium* sp. were isolated from about 40% of the fish samples (Table 2). *Edwardsiella ictaluri* was identified in fish samples with gas bubbles and fluid in the stomach/gut.

*Aeromonas hydrophila* was isolated from diseased fish with gross signs of hemorrhage on the mouth and around the eyes, exophthalmus, hemorrhagic fins and caudal peduncle, and a distended liver (Fig. 1). *Aeromonas hydrophila* colonies were 1-1.5 mm in diameter and cream colored on blood agar medium with beta hemolysis. The colony was surrounded by a clear zone in which few or no intact erythrocytes were apparent. On Rimler-Shotts medium, the colony was round and yellow, 1-1.5 mm in diameter, with rapid growth in 18-24 h. The bacterium was gram negative.

*Clostridium* sp. was isolated from diseased fish with hemorrhage along the ventral fin base, swollen kidney and spleen, and many necrotic nodules in the liver, kidney, and spleen (Fig. 2). *Clostridium* sp. grew well on Wilson-Blair agar under strictly anaerobic conditions (Fig. 3). The colony was black, 2-3 mm in diameter, and circular. The bacteria were gram positive, 2.0-4.0 µm

Table 2. Number and percent of positively identified bacteria isolations from tra catfish (n = 49) with hemorrhage disease from four provinces in Vietnam.

Bacteria	Liver	Kidney	Total
<i>Aeromonas hydrophila</i>	15 (30.6%)	19 (38.8%)	19 (38.8%)
<i>Aeromonas caviae</i>	1 (2.0%)	0	1 (2.0%)
<i>Aeromonas sobria</i>	2 (4.1%)	0	2 (4.1%)
<i>Edwardsiella ictaluri</i>	2 (4.1%)	0	2 (4.1%)
<i>Clostridium</i> sp.	20 (40.8%)	19 (38.8%)	20 (40.8%)

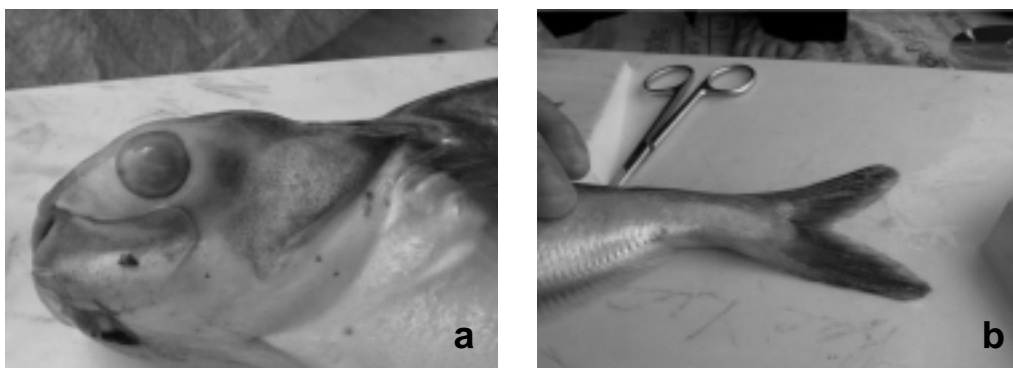


Fig. 1. Gross signs of (a) hemorrhage around the eyes and exophthalmus and (b) hemorrhagic fins and caudal peduncle in tra catfish (*Pangasianodon hypophthalmus*) infected by *Aeromonas hydrophila*.

x 1.0-1.5  $\mu\text{m}$  (length varied according to stage of growth), in pairs or chains, straight rods with endospores, and motile with peritrichous flagella.

*Edwardsiella ictaluri* was isolated from fish exhibiting gas bubbles and fluid in the stomach and gut, and a bad smell (Fig. 4). The bacteria were gram negative. On blood agar medium, the colonies were cream colored, surrounded by a zone of intact but discolored erythrocytes, and 1-1.5 mm in diameter after 36-48 h incubation at 28-30°C. On Mac Conkey agar, the colonies were light pink.

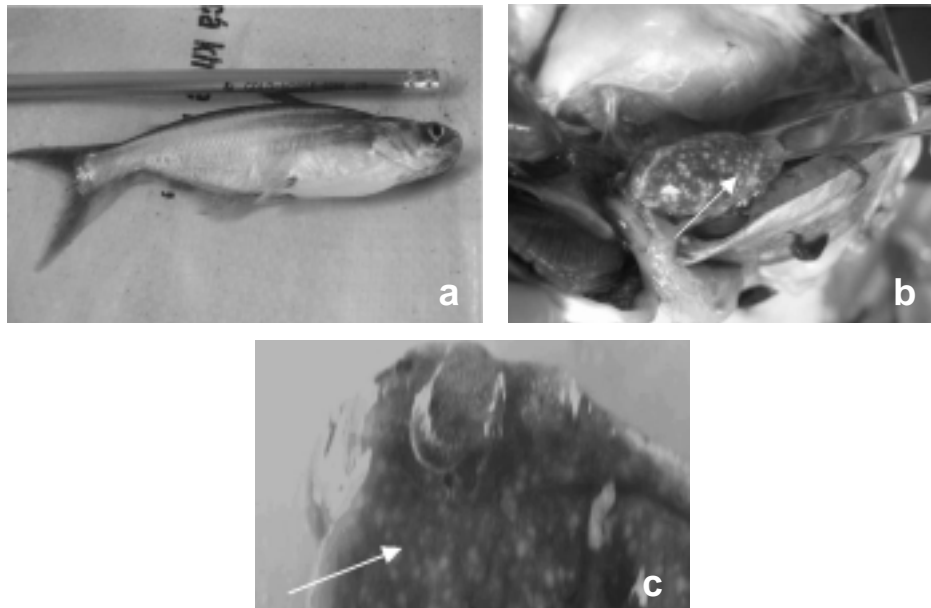


Fig. 2. Gross signs of (a) hemorrhage along the ventral fin base and (b,c) white nodules in the kidney of tra catfish (*Pangasianodon hypophthalmus*) infected by *Clostridium* sp.

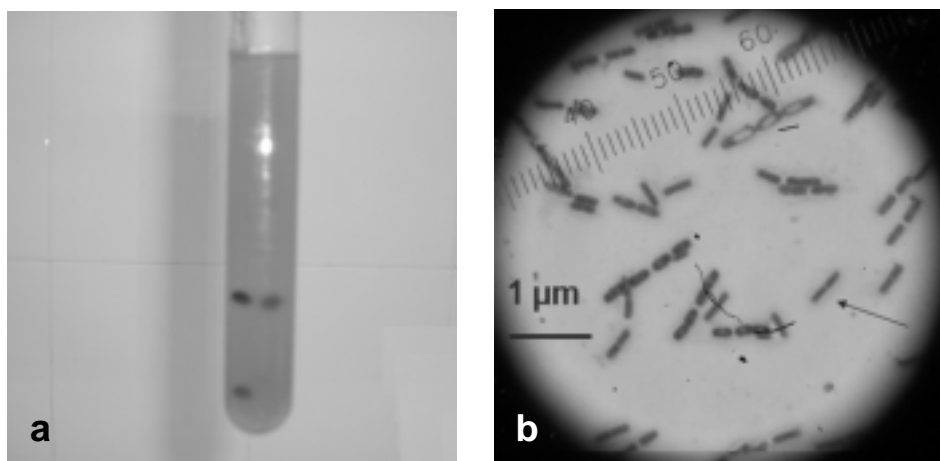


Fig. 3. Colony of *Clostridium* sp. cultured in (a) Wilson Blair agar and (b) Gram-stained bacteria (x 100).



Fig. 4. A gas bubble in the stomach and fluid in the gut of tra catfish infected with *Edwardsiella ictaluri*.

**Histopathology.** Kidney, liver, and spleen cells were severely damaged (Fig. 5). Degenerative changes consisting of focal necrosis and hemorrhage were detected in the spleen and kidney tubules. Cells were incoherent and bacterial cells were observed in the tubules. The tissues of the kidney and liver showed cellular degeneration (Fig. 6). White nodules in the liver and kidney showed the presence of bacterial infection.

**Virus isolation.** None of the 39 pooled tissue filtrate samples inoculated onto BF-2 cells in 24-well plates and incubated at 27°C exhibited CPE.

**Transmission electron microscopy (TEM).** Abundant bacteria were detected by TEM in white nodules of the kidney and spleen of infected fish (Fig. 7). The bacteria fell into two size ranges: 2.0-4.0 x 1.0-1.5 µm and 3.5-4.0 x 0.8-1.0 µm, suggesting that the fish were infected by two or three different kinds of bacteria. The bacteria were straight plump rods with rounded ends, located outside or inside the cells. Virions were seen in the spleen. Icosahedral double capsid structures were observed in negatively-stained preparations of the infected tissues (Fig. 8). The virions had an inner diameter about of 40-50 nm and an outer diameter of 70-75 nm.

**Fish challenge tests.** After injection with *A. hydrophila*, test fish showed signs of swollen anus and hemorrhage of the body, mouth, and fin bases. Internally, the liver and kidney were hemorrhagic, swollen, and necrotic. Focal necrosis of the liver was also noted. Seven days after bacterial injection, 73.3% of the fish were sick and 36.6% had died (Table 3). No further mortality devel-

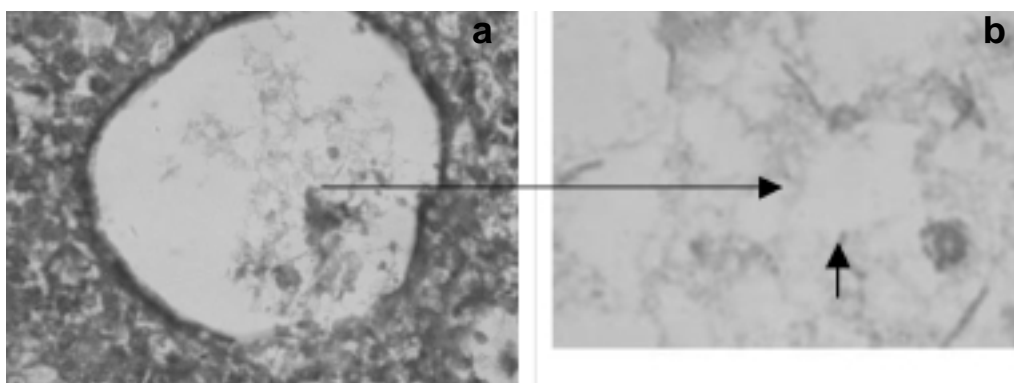


Fig. 5. (a) Section of the kidney of infected tra catfish showing necrosis and cellular degeneration with (b) bacterial cells (arrow) as seen in Wright-Giemsa, x 100.

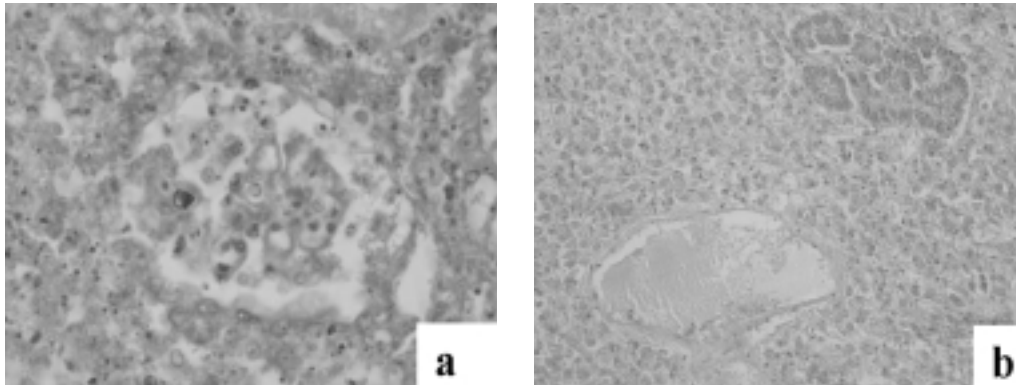


Fig. 6. Section of a white nodule in the (a) kidney (H&E x 40) and (b) liver (H&E x 10) of an infected tra catfish showing cellular degeneration in necrotic areas.

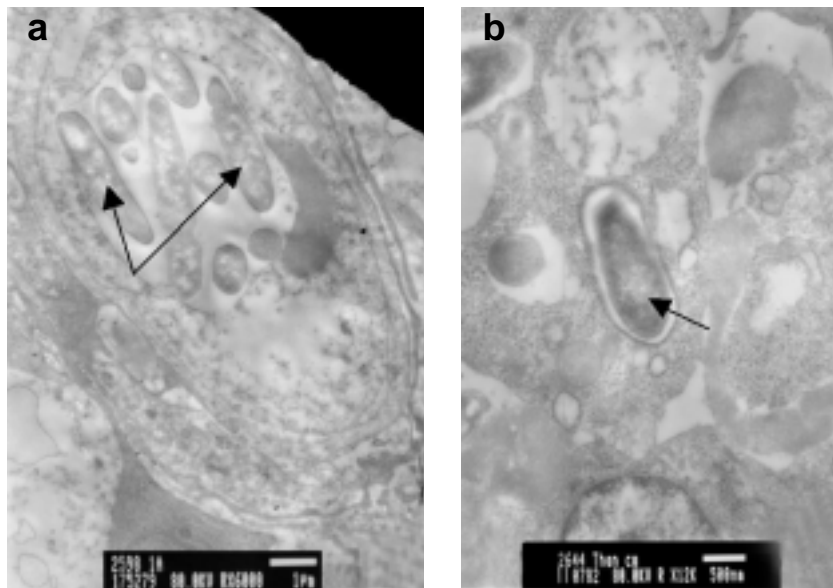


Fig. 7. Transmission electron micrograph of (a) bacteria in kidney tissue (bar = 1 µm) and (b) the bacterial cell (bar = 500 nm); arrows point to bacteria cells.

oped after day 7 and *A. hydrophila* was re-isolated from the affected organs of the challenged fish.

Fish challenged with *E. ictaluri* showed clinical signs of petechial hemorrhage around the eyes. Internal signs consisted of very soft (necrotic) liver and kidney with gas bubbles in the stomach. Seven days after injection, the percentage of sick fish was 66.7% and mortality was 33.3%. Beyond seven days, there was no further morbidity or mortality. *Edwardsiella ictaluri* was re-isolated from the liver, spleen, and kidney of the challenged fish.

Three days after challenge with *Clostridium* sp., the fish were normal and fed well. However,



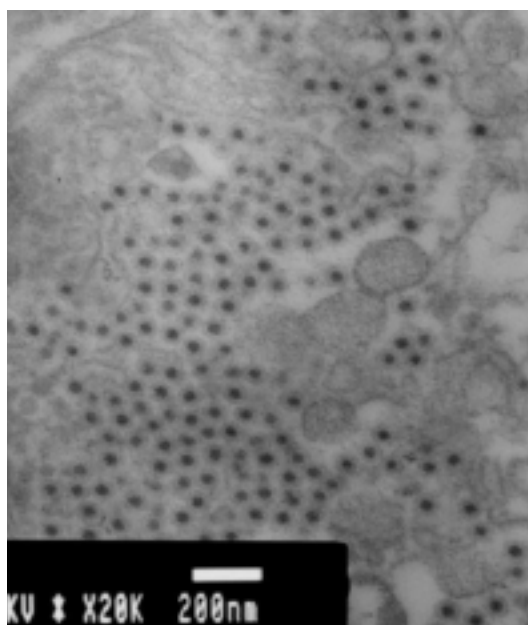


Fig. 8. Transmission electron micrograph of the icosahedral reovirus-like particles in spleen cells of naturally-infected tra catfish (bar = 200 nm).

infected fish began to exhibit disoriented swimming with the head protruding above the water surface and a lack of appetite. All the challenged fish became infected and died within seven days. Fish injected with *Clostridium* sp. manifested swollen kidneys, livers, and spleens with severe hemorrhage and many white nodules. They had petechial hemorrhages on the tail and along the ventral fin base. Dead fish had soft and swollen kidneys and spleens with multifocal white nodules. The latter signs were more often observed and more severe in the kidney than in the liver. Bacteria re-isolated from these organs were identified as *Clostridium* sp.

#### Discussion

Hemorrhage disease of tra catfish in Vietnam commonly occurs at the beginning of the rainy season. In this study, five species of bacteria were isolated from tra catfish manifesting clinical signs of hemorrhage or clinical signs of hemorrhage associated with white nodular lesions in the internal organs or gas bubbles in the stomach and a gut that exuded a foul smell.

Nineteen of the 49 naturally-infected fish samples were infected with *A. hydrophila*. *Aeromonas* is an opportunistic pathogen, especially in intensive culture when many stress conditions, i.e., poor environment and high stocking density, can affect fish. Then the fish become susceptible to pathogens and disease breaks out. The challenge test confirmed that *A. hydrophila* induced clinical signs of hemorrhagic and swollen anus, and hemorrhage of the body, mouth, and fin bases. These differed from the hemorrhage alongside the ventral fin base induced by *Clostridium* sp.

*Clostridium* spp. are numerous in the intestinal tract of man and animals. Its pathogenicity is associated with the secretion of potent toxins. *Clostridium botulinum* was detected as a pathogen that caused poisoning in rainbow trout in Britain although no typical signs were reported.

Table 3. Cumulative morbidity and mortality of tra catfish after intraperitoneal injection with one of three bacteria (n = 30 fish per treatment).

Treatment	Day 3		Day 6		Day 7	
	Sick	Dead	Sick	Dead	Sick	Dead
Control	0	0	0	0	0	1
<i>Aeromonas hydrophila</i>	4 (13.3%)	0	13 (43.3%)	8 (26.7%)	22 (73.3%)	11 (36.7%)
<i>Edwardsiella ictaluri</i>	4 (13.3%)	0	20 (66.7%)	10 (33.3%)	20 (66.7%)	10 (33.3%)
<i>Clostridium</i> sp.	0	0	30 (100%)	17 (56.7%)	30 (100%)	30 (100%)

ed on the sick fish (Cann and Taylor, 1982). In this study, all challenged fish developed disease and died within 7 days post injection due, perhaps, to the toxin of *Clostridium* sp. Clinical signs in the challenged fish were the same as in naturally-infected hemorrhage disease of tra catfish except for the absence of hemorrhage in the body, mouth, liver, and kidney.

*Edwardsiella ictaluri* caused enteric septicemia of catfish (ESC) in channel catfish in the USA (Hawke et al., 1998). Diseased fish developed petechial hemorrhage of the skin and the body cavity was sometimes filled with a cloudy, bloody, or (rarely) clear yellow fluid. The kidney and spleen were enlarged, the spleen was dark red and the liver was either pale or mottled with congestion (Plumb, 1993). *Edwardsiella ictaluri* was reported to cause multifocal white lesions in the liver, spleen, and kidney of tra catfish in Vietnam (Crumlish et al., 2002). In this study, *E. ictaluri* were isolated from diseased tra catfish but results of the challenge test were inconclusive compared to a previous report (Crumlish et al., 2002).

TEM showed many rod-shaped bacteria with rounded ends that were 0.8-1.0  $\mu\text{m}$  x 3.5-4.0  $\mu\text{m}$ , similar in size to *A. hydrophila* (1.0 x 4.0  $\mu\text{m}$ ) and *E. ictaluri* (1.0 x 2-3  $\mu\text{m}$ ) as reported by Plumb (1993). *Edwardsiella ictaluri* from tra catfish of Vietnam were typical, but had greater variability in length and width, often with very large rods (Crumlish et al., 2002).

Channel catfish virus (CCV) and channel catfish reovirus (CRV) were earlier reported in channel catfish (Wolf, 1988). These were isolated in cell cultures using channel catfish ovary (CCO) and brown bullhead (BB) cell lines. CRV can also replicate in cells of Chinook salmon embryo (CHSE-214). CCV can be isolated from very young catfish. It is a member of *Herpesviridae*, provisionally designated *Ictalurid herpesvirus 1*, and affects channel catfish in the USA. Outbreaks of CCV disease result in high mortality among fry and juvenile catfish. Diseased fish demonstrate ascites, exophthalmia, and hemorrhage in fins and musculature. Histologically, the most remarkable damage occurs in the kidney with extensive necrosis of renal tubules and interstitial tissue. Affected fish show signs of a hemorrhagic disease and kidney dysfunction. The abdomen is swollen and, in some, the vein may be distended. Fin bases, especially of the ventral fins, the abdomen, and the caudal peduncle are typically hemorrhagic. Such fish develop exophthalmia, with some fish showing yellowish external lesions indicating secondary invasion by *Flexibacter columnaris* or *A. hydrophila*. Internally, the spleen is usually enlarged and dark, and the kidney and liver are hemorrhagic or flecked with petechiae (Wolf, 1988). CRV is known only from channel catfish in southern California. The virus does not cause severe disease (Wolf, 1988). CRV is doubly encapsidated; the inner form measures 55 nm and the outer form 75 nm. Replication of CRV occurs at 20-30°C, and the optimum is about 25°C. Maximal infectivity (tissue culture infective dose; TCID) is about  $10^{8.1}$  TCID<sub>50</sub>/ml by BB cells and  $10^{7.3}$  TCID<sub>50</sub>/ml by CCO cells (Wolf, 1988).

In our study, virus was found in sick fish manifesting petechial hemorrhage around the eyes and pale gills, accompanied with lesions in the liver, kidney, and spleen. The virions observed by TEM were similar to CRV in size and shape. Hence, the particle is tentatively named reovirus-like virus of tra catfish. Unfortunately, no CPE were observed in BF-2, even after two cell culture passages. Future research to isolate the virus using more sensitive cell lines and bioassay of healthy tra catfish should be conducted.

By and large, this study showed that *A. hydrophila* and *Clostridium* sp. were the predominant bacteria in the 49 tra catfish that manifested hemorrhage disease. While the *A. hydrophila* and *E. ictaluri* challenge tests reproduced hemorrhagic clinical signs of the disease in about two-thirds of the test fish with approximately one-third mortality, infection with *Clostridium* sp. induced some hemorrhagic clinical signs, development of nodules in the liver, kidney, and spleen, and mortality in 100% of the test fish. Although the challenge tests induced clinical signs similar to those in naturally-infected tra catfish, no evidence points exclusively to a single pathologic effect of any of the three test bacteria. TEM analyses showed the presence of a virus and multiple bacterial sizes in the infected fish. Hence, further confirmatory challenge tests are suggested using single or multiple bacteria with or without the virus.

### Conclusions

This study shows that hemorrhage disease of tra catfish may be caused by either *A. hydrophila* or *Clostridium* sp. or *E. ictalurus*. It is also possible that a combination of these bacteria, with or without a virus, is present in hemorrhage disease of tra catfish. The role of each pathogen in a multiple pathogen infection bioassay needs to be confirmed in future studies.

### Acknowledgements

We thank the Government of Japan Trust Fund Project (GOP-TF) and SEAFDEC/AQD for financial support through Drs. Koichi Okuzawa, Hiroshi Ogata, and Joebert D. Toledo. We also thank Dr. Gilda Lio-Po for her painstaking review of the manuscript and Ma Tu Lan, Truong Hong Viet, Pham Vo Ngoc Anh, and Nguyen Van Huy for their technical assistance. Likewise, we sincerely thank the farmers who provided the samples.

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